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EXHIBIT A

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Covalent attachment of DNA oligonucleotides to glass

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ABSTRACT

A method for synthesizing DNA directly on glass is presented. The process is based on pretreatment of glass with boiling hydrochloric acid, thus exposing the hydroxyl groups of the glass. Phosphite-triester chemistry was used to directly attach the first nucleotide to a glass plate via a covalent bond between the hydroxyl group of the glass and the phosphate group of the protected deoxyribonucleotide. Standard molecular biology procedures, such as ligation and restriction digest, were efficiently performed on the glass synthesized oligonucleotide, with the added benefit of extreme ease of handling.

Attachment of DNA to a solid support is of interest for many biotechnological and molecular biology applications (1–3). Presently DNA is synthesized on a solid support by the incorporation of a spacer between the solid support (usually Controlled Pore Glass–CPG) and the first nucleotide. This technology is highly efficient and is advantageous when the synthesized DNA needs to be detached from the support, which is usually the case. However, in many cases a strong bond between the support and the DNA molecule is required (4,5). In such cases the lability of the ester bond between the spacer and the DNA molecule becomes a drawback. Here we report on a simple and efficient method for synthesizing DNA directly on glass plates.

To replace the weak ester bond between the support and first nucleotide, we employed phosphite-triester chemistry (6,7) to directly attach the first nucleotide to a glass plate. This process forms a covalent bond between the hydroxyl group of the glass and the phosphate group of the protected deoxyribonucleotide. This covalent bond is similar in structure and strength to the phosphodiester bonds within the DNA molecule. Additional chemical synthesis of more deoxyribonucleosides yields a deoxyoligonucleotide of the required length. The oligodeoxynucleotide remained intact and attached to the glass following deprotection. Standard molecular biology procedures, such as ligation and restriction, can be efficiently performed on the deoxyoligonucleotide that remained attached to the glass support. The macroscopic solid support makes it extremely easy to manipulate the DNA throughout the various reactions.

The synthesis was performed on 9 mm^2 glass pieces cut from a microscopic slide (Knittal, Germany) preconditioned by immersion in 32% HCl at 100°C for 3 h, in order to expose the hydroxyl group of the glass. The glass pieces were packed into a standard automated DNA synthesis column (~10 pieces per column). The phosphorylation was carried out with an automated DNA synthesizer (Applied Biosystems, Foster City, CA) with a minor variation of the manufacturer's protocol (15 min phosphorylation step). The efficiency of oligonucleotide chain growth was monitored by measuring the visible absorbance at 498 nm of the released DMT protecting group in acetonitrile solution. The yield of each cycle was 99% (from the 6th cycle on) with an estimated surface density of 10^{14} (oligonucleotide chains)/(cm^2 of glass area). The final deprotection was done manually by incubating the glass pieces for 6 h in 25% aqueous NH_4OH at 55°C. Absorption measurements of the NH_4OH supernatant at 260 nm showed no loss of DNA from the glass.

To check whether additional double stranded DNA fragments can be ligated and subsequently digested, a dsDNA fragment was prepared as follows. Lambda DNA was digested with *PmeI* (blunt end cutter) and *AflII* (which leaves a 5′-TTAA-3′ overhang). A 2 kb fragment was excised and purified from 0.8% agarose gel. The fragment was extended with α-^{32}P labeled deoxythymidine (dTTP, Amersham, 3000 Ci/mmol) and Klenow polymerase resulting in 5′-T-T-3′ overhang. ^{32}P labeling occurred also at the 3′ blunt end due to the exchange of terminal thymidine by the 3′→5′ exonuclease activity of the Klenow enzyme. The sequence synthesized on glass corresponded to the antisense of the (–47) Universal primer (NE Biolabs) with two additional deoxyadenosine residues at the 5′ end, resulting in a total 26mer oligonucleotide grown on the glass. This oligonucleotide was phosphorylated with T4 polynucleotide kinase. The lambda DNA fragment together with the complementary (–47) Universal primer (NE Biolabs) were ligated to the strand synthesized on the glass to create a complete double stranded DNA molecule. The ligation reaction was monitored by Cherenkov emission. Efficiency was estimated to be 10^{-4}% of the sites that were synthesized on the glass (10^8 fragments/cm^2). Following ligation, the glass was washed three times with water, incubated with 0.2 M NaCl for 15 min to remove nonspecifically bound DNA, and washed again with water. No decrease in Cherenkov counts following washes was observed. As a control, an identical reaction without the ligase was done. The Cherenkov counts in the control (represent-

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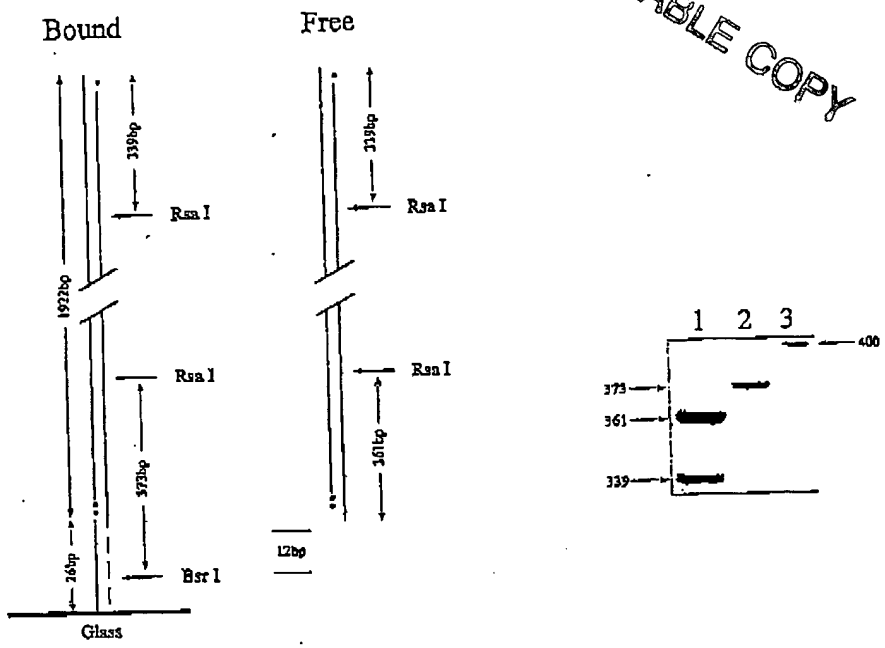

Figure 1. The 1922 bp lambda fragment was labeled (denoted by an asterisk) with α-dTTP at both ends and ligated to the glass attached oligonucleotide together with an oligonucleotide complimentary to the attached strand (dashed line). The bound and free (unligated) fragments were digested separately with *Rsa*I. The DNA released from the bound fragment was removed as described, leaving 373 bp attached to the glass. The bound DNA was further digested with *Bst*I. The digested DNA was separated on 7% denaturing polyacrylamide gel and exposed to FUJI RX X-ray film with an intensifying screen. The two fragments (339 and 361 bp) in lane 1 correspond to the two terminal labeled fragments of the *Rsa*I digested free DNA (note that the relative intensity of the two bands is consistent with the incorporation of one and two radionucleotides). Lane 2 represents the *Bst*I digested DNA. The 373 bp band is 12 bp longer than the corresponding 361 bp band due to ligation of the lambda fragment to the glass synthesized DNA. Labeled DNA ladder (GIBCO BRL Cat. No. 10068-021) was run in lane 3. The 400 bp fragment is shown. An independent calibration of fragment lengths was obtained by a fit of the six ladder bands. The lengths of all shown fragments fully agreed with their calibrated lengths.

ing the nonspecific binding) were 10-fold less. The ligated DNA was cut with *Mse*I, which cuts between the two radioactively labeled thymidines at the ligation site. The c.p.m. count from the glass decreased by 65% following *Mse*I digestion, indicating complete removal of the ligated DNA (taking into account the blunt end labeling).

In a separate experiment, the same DNA fragment ligated to glass was cut with *Rsa*I, which cuts at 361 bp from the ligation site. A 30% decrease in the Cherenkov signal was observed, consistent with the removal of the blunt end labeled DNA. After washing, the ligated DNA was further cut with *Bst*I which cuts 12 bp below the ligation site (within the sequence originally synthesized directly on the glass). A 60% decrease in Cherenkov counts was observed, showing that the DNA fragment was indeed ligated to the DNA synthesized on glass. The reduction in the efficiency of the *Bst*I activity was probably a result of the proximity of the digestion site to the glass. The digestion products were separated in 7% denaturing polyacrylamide gel. As shown in Figure 1, the *Bst*I digest resulted in a 12 bp increase in length of the ligated DNA, further proving the ligation step.

In summary, these results confirm correct synthesis, ligation and digestion steps of glass attached DNA. DNA synthesis on pretreated glass is highly efficient and has several advantages over the conventional CPG-initiated synthesis: the ester bond between the glass and the DNA is of similar structure and strength to the phosphodiester bonds in DNA; the glass-attached DNA is extremely easy to handle and it is well suited for all conventional procedures in molecular biology with the added value of improved purity and reduction in time due to fast buffer changes.

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